

LACTOSE OXIDASE: A PRESERVATIVE OF DAIRY PRODUCTS

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ABSTRACT

Lactose oxidase (LO) oxidizes lactose to lactobionic acid, producing H_2O_2 that could act as an antimicrobial and activate the lactoperoxidase system (LPS), an antimicrobial system present in milk. We evaluated the antimicrobial effect of LO alone and with the components of the LPS: SCN^- and lactoperoxidase. An increase in LO caused higher reductions of *Pseudomonas fragi* in pasteurized milk. A total bacterial growth curve in raw milk showed a longer lag phase and lower counts when the LPS was activated by LO compared to the control. The antimicrobial effect of LO against foodborne pathogens and spoilage microorganisms was evaluated through an overlay inhibition assay. LO was applied in different media to evaluate the effect of lactose and SCN^- . All the indicators were inhibited by LO in presence of lactose. However, SCN^- had no effect on the inhibition. The antimicrobial effect was attributed to H_2O_2 produced from the oxidation of lactose.

BIOGRAPHICAL SKETCH

Sofía is from San José, Costa Rica. She graduated from the University of Costa Rica with a bachelor's degree in Food Engineering. During her studies, she served as teaching assistant for sensory analysis, food microbiology, and organic chemistry. For her thesis, Sofia did research on the effect of sonication of raw milk on processing parameters, quality and safety of cheese. She also participated successfully in product development competitions and did an internship in the Research and Development Department of Dos Pinos, a Costa Rican dairy cooperative. Early in her undergraduate studies she decided to follow a career in dairy science, intrigued by the versatile field that relies on the integration of chemistry, microbiology, and engineering in order to produce desirable products.

In 2016, Sofía joined Alcaine Research Group as a master's student. Her research focused on dairy biopreservation. Specifically, her work involved investigating the application of lactose oxidase as an enzymatic preservative to improve the shelf life and safety of dairy products. In addition to research, Sofía is a trained dairy sensory panelist, where she has participated in national competitions and is currently the assistant coach of Cornell's Dairy Products Sensory Evaluation Team. Sofía was an active participant in the Food Science Department. She served as vice-president of the Food Science Graduate Student Organization and participated in a product development competition.

Upon graduation, Sofía aspires to continue serving the dairy industry by developing products and applying new technologies in alignment with consumer needs, as well improving consumer education to promote dairy products consumption. She aims to innovate and help in transferring knowledge to the industry, not only to improve the processing and quality of food, but also to generate new opportunities for dairy producers.

To my mom and dad for their unconditional love and support

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CHAPTER 1

LACTOSE OXIDASE: A NOVEL ACTIVATOR OF THE LACTOPEROXIDASE SYSTEM IN MILK FOR IMPROVED SHELF-LIFE

ABSTRACT

The lactoperoxidase system (LPS) is an antimicrobial system naturally present in milk, that is activated by H_2O_2 , and has been used to inhibit microbial outgrowth in raw milk in areas where refrigeration is not viable. This study evaluated lactose oxidase (LO) as a novel activator of the LPS. LO oxidizes lactose and produces H_2O_2 needed for the activation of the LPS. The antimicrobial effect of different concentrations of LO with and without and without components of the LPS system, NaSCN and lactoperoxidase (LP), was evaluated in model systems and then applied in pasteurized milk and raw milk. In general, an increase in LO caused higher reductions of *P. fragi* and treatments were more effective at 6 °C than at 21 °C. The LO solution at 0.12 and 1.2 g/L showed a significantly higher microbial reduction than the control when added both alone and combined with the LPS components. At 21 °C, treatments with 1.2 g/L of LO solution achieved a reduction of >2.93 log CFU/mL, but at lower levels there was not a significant reduction from the control. Higher concentrations of NaSCN led to a greater *P. fragi* reduction at both temperatures when LO was added alone but not when combined with LP. In pasteurized milk, the LO solution at 0.12 g/L caused a reduction of *P. fragi* within 24 h of ~1.4 log when added alone and of ~2.7-log when combined with LP and NaSCN. Bacterial counts remained at significantly lower levels than the control during storage and the NaSCN- supplemented milk exhibited a ~ 6-log difference from the control by day 7. The total bacterial growth curve in raw milk showed a longer lag phase when the LPS was activated by LO (11.3 h) compared to the control (4.0 h) but

it was not different from the recommended method (9.4 h). However, the total bacterial count after 24 h for the LO-NaSCN-treated sample (5.3 log CFU/mL) was significantly lower compared to the control (7.2 log CFU/mL) and the recommended method (6.1 log CFU/mL). Results from this study suggest that LO is an alternative source of H₂O₂ that enhances the microbial inhibition achieved by the LPS. LO could be used to develop enzyme-based preservation technologies for applications where cold chain access is limited. This enzymatic approach to improve the shelf-life of dairy products also represents a novel option for a clean label spoilage control.

INTRODUCTION

Milk is a nutrient-dense food, a characteristic that makes it a popular product in human diet but it also means it is very susceptible to microbial spoilage. Different methods, such as refrigeration and heat treatments, have been used to limit spoilage of fluid milk along the supply chain. The lactoperoxidase system (LPS), an antimicrobial system endemic to milk, represents an alternative, enzymatic-based preservation method to inhibit microbial growth and extend raw milk shelf life (Seifu et al., 2005). The use of this endogenous antimicrobial system is especially important in developing countries and rural areas, where refrigeration of milk before processing or consumption is not feasible due to its high cost, lack of electricity, or difficulties with maintenance and repair of the equipment (Seifu et al., 2005). The LPS enables extended milk storage at ambient temperatures and has shown to be more profitable than cooling for small scale dairy producers, and thereby improving their milk marketability, income, and employment opportunities (FAO/WHO, 2006).

LPS consists of three components: lactoperoxidase (LP), thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2). The enzyme catalyzes the peroxidation of SCN^- into hypothiocyanite (OSCN^-) which is the main antimicrobial component, in combination with other intermediary products (De Wit and Van Hooydonk, 1996). The concentration of both SCN^- and H_2O_2 in the milk impact the magnitude of the antimicrobial effect. LPS has been reported to have a bacteriostatic effect against Gram-positive bacteria such as streptococci and lactobacilli, as well as a bactericidal effect against Gram-negative bacteria like pseudomonads and coliforms (Kussendrager and van Hooijdonk, 2007).

LP is a heat sensitive enzyme; its residual activity after HTST pasteurization is approximately 70 % and UHT pasteurization inactivates it completely (Barret et al., 1999). The average concentration of LP in raw milk is 30 mg/L, while SCN^- can vary between 1 and 15 mg/L depending on factors such as animal feed and breed of cow (Kussendrager and van Hooijdonk, 2007). The natural concentration of H_2O_2 in milk, however, is not sufficient to activate the antimicrobial system. Therefore, methods to generate this substrate are required, and have included: i) the addition of sodium carbonate peroxyhydrate; ii) the activation of H_2O_2 producing systems such as glucose-glucose oxidase and xanthine-xanthine oxidase; and iii) the endogenous production of H_2O_2 by catalase negative microorganisms (Adolphe et al., 2006, FAO/WHO, 2006).

Lactose oxidase (LO) oxidizes lactose to lactobionic acid with the concurrent reduction of O_2 to H_2O_2 (Nordkvist et al., 2007). The enzyme used in this study was cloned from *Microdochium nivale* and expressed in *Fusarium venenatum*. Ahmad et al. (2004) concluded that it is safe to use lactose oxidase in food for human consumption. Currently, LO is primarily used for the production lactobionic acid, a compound used for the preservation of tissues by the medical industry due to its metal-chelating properties. Potential uses for the acid in the food industry are also as metal chelator, protein and fat replacer in cheese, and flavor enhancer (Ahmad et al., 2004). Its potential as an activator of LPS or as an antimicrobial agent in dairy products has not been studied.

The first part of this study explored the effect of the concentration of lactose oxidase – alone and as an activator of the LPS – and of thiocyanate on the outgrowth of *Pseudomonas fragi* in UHT milk during storage at 6 °C and 21 °C. *Pseudomonas* spp. are one the most common spoilage

microorganisms associated with milk and dairy products due in part to their ability to grow under refrigeration conditions. Stellato et al. (2017) found that *P. fragi* and *P. fluorescens* are the most abundant species in dairy samples and processing plants. The generated data was then used as reference to select the concentration of lactose oxidase to use in two applications: (1) as antimicrobial in pasteurized milk spiked with *P. fragi* to mimic post-pasteurization contamination, and (2) as an activator of the LPS in raw milk in comparison with the method described by FAO/WHO (1991) to activate the system. The results of this study describe the application of LO as novel source of H₂O₂ that utilizes naturally present constituents of milk, lactose and oxygen, to enhance the microbial inhibition achieved by the LPS. LO could be used to create enzyme-based preservation technologies to improve the shelf-life of dairy products or for applications where cold chain access is limited.

MATERIALS AND METHODS

Inoculum preparation

A frozen stock of a *Pseudomonas fragi* isolated from pasteurized milk was obtained from the Cornell University Food Safety Laboratory isolate collection. The frozen culture was streaked onto brain heart infusion agar (BHI; Difco, BD Diagnostics, Franklin Lakes, NJ) and incubated at $32 \pm 1^\circ\text{C}$ for 24 h. An individual colony was then used to inoculate 5 mL of BHI broth, followed by 18 h of incubation at $32 \pm 1^\circ\text{C}$. A 1-mL aliquot of the overnight culture was transferred to a 1.5 mL Eppendorf tube and centrifuged for 1 min at 14,000 g. The supernatant was removed and the cell pellet was re-suspended in 1 mL of phosphate buffered saline solution (Weber Scientific, Trenton, NJ) and then used as the inoculum.

Evaluation of the concentration of lactose oxidase

One liter of ultra-pasteurized (UHT) skim milk (Parmalat USA Corp, Grand Rapids, MI) was inoculated with *P. fragi* to obtain an approximate load of 4 log CFU/mL. The initial bacterial concentration was confirmed by plating the inoculated milk immediately after inoculation. Bovine lactoperoxidase (Sigma-Aldrich, Co., St. Louis, MO) and NaSCN (VWR International, LLC, Solon, OH) were added to 330 mL of inoculated milk to a final concentration of 30 mg/L and 14 mg/L, respectively. NaSCN was added as a solution (4.6 w/v %) previously prepared in distilled water and filter-sterilized through a surfactant-free cellulose acetate (SFCA) filter of 0.20 μm pore size (Corning Incorporated, Corning, NY). An enzymatic solution of lactose oxidase

(LactoYIELD[®], Chr. Hansen, Milwaukee, WI) was then added at concentrations of 0.012, 0.12 and 1.2 g/L to individual 70-mL volumes of both the inoculated milk and the NaSCN supplemented milk, to obtain a total of six different treatments. Samples were aliquoted into separate test tubes for every time point and were then incubated at $6 \pm 1^{\circ}\text{C}$ or $21 \pm 1^{\circ}\text{C}$. Samples were plated for microbiological load after 1, 2, 4, and 7 days of storage. The experiment was carried out in triplicate and inoculated milk with no LPS/NaSCN supplementation or added enzyme was used as the control.

Evaluation of the concentration of thiocyanate

One liter of ultra-pasteurized (UHT) skim milk (Parmalat USA Corp, Grand Rapids, MI) was inoculated with *P. fragi* to obtain an approximate load of 4 log CFU/mL. The initial bacterial concentration was confirmed by plating the inoculated milk immediately after inoculation. Bovine lactoperoxidase (Sigma-Aldrich, Co., St. Louis, MO) was added to 330 mL of the inoculated milk to a final concentration of 30 mg/L. NaSCN was then added to individual 70-mL aliquots of both milk fractions to reach final concentrations of 14, 140 and 1400 mg/L; followed by the addition of the lactose oxidase solution (LactoYIELD[®], Chr. Hansen, Milwaukee, WI) at 0.12 g/L to all milk samples to obtain a total of six different treatments. NaSCN was added as a solution (32.7 w/v %) previously prepared in distilled water and filter-sterilized through a SFCA 0.20 μm pore size filter. Samples were aliquoted into separate test tubes that were then incubated at $6 \pm 1^{\circ}\text{C}$ or $21 \pm 1^{\circ}\text{C}$. Samples were plated for microbiological load after 1, 2, 4, and 7 days of storage. The experiment was carried out in triplicate and inoculated milk with no LPS/NaSCN supplementation or added enzyme was used as the control.

Application of lactose oxidase in pasteurized milk

One liter of pasteurized skim milk (Cornell Dairy, Ithaca, NY) was inoculated with *P. fragi* to obtain an approximate load of 4 log CFU/mL. Lactose oxidase (LactoYIELD[®], Chr. Hansen, Milwaukee, WI) was incorporated into individual 70-mL samples at concentrations of 0.006, 0.012 and 0.12 g/L. NaSCN solution was added to 300 mL of inoculated milk to a concentration of 14 mg/L, and afterwards, lactose oxidase was added to three 70-mL samples at the same three concentrations previously mentioned. NaSCN was added as a solution (4.6 w/v %) previously prepared in distilled water and filter-sterilized through a SFCA 0.20 µm pore size filter. Samples were aliquoted into separate test tubes that were then incubated at $6 \pm 1^{\circ}\text{C}$. Samples were plated for microbiological load after 0, 1, 2, 4, and 7 days of storage. The pH was measured for all samples at the same time points. The experiment was carried out in triplicate and inoculated milk with no LPS/NaSCN supplementation or added enzyme was used as the control.

Application of lactose oxidase as raw milk preservative

Whole raw milk was collected from Cornell's Teaching Barn (Cornell University, Ithaca, NY) within 1 hour from the time of milking. The bottle containing the milk was inverted 25 times before dividing it in three separate 1-L samples. The first sample was prepared following the process described by FAO/WHO (1991), which consists on the addition of 14 mg/L of NaSCN and 30 mg/L of sodium percarbonate. NaSCN was added as a solution (0.028 g/mL) prepared in distilled water and filter-sterilized through a SFCA 0.20 µm pore size filter. A solution of sodium percarbonate (0.06 g/mL) in sterilized distilled-deionized water was immediately added after being

prepared. The second milk sample was prepared adding NaSCN and lactose oxidase (LactoYIELD[®], Chr. Hansen, Milwaukee, WI) solutions to reach final concentrations of 14 mg/L and 0.12 g/L, respectively. The third sample was prepared adding the lactose oxidase solution at a concentration of 0.12 g/L. All samples were incubated at 21 ± 1 °C for 24 h and a total microbial count was carried out every two hours for the first 12 hours of incubation, and every four hours for the remaining time. The pH was measured for all samples at the same time points. The experiment was carried out in triplicate and inoculated milk with no supplementation or added enzyme was taken as the control.

Microbiological analysis

Serial dilutions of the milk samples were prepared in phosphate buffer and spread in duplicate on Standard Plate Count agar. Plates were incubated at 32 ± 1 °C for 48 h before enumeration.

Statistical analysis

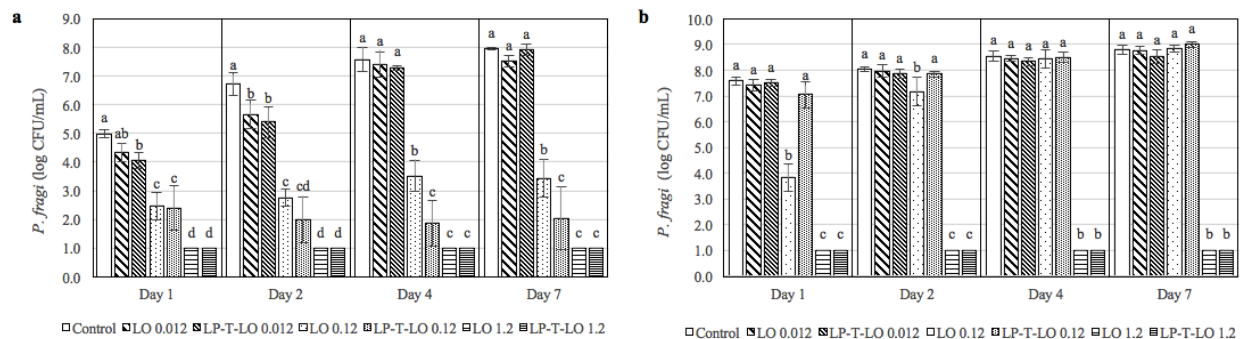
All statistical analyses were performed using R (version 3.4.1, R Development Core Team, Vienna, Austria). An ANOVA and a Tukey's honest significant difference test were performed individually for each time point to compare the log differences in microbial counts between treatments corresponding to the evaluation of the concentration of LO and NaSCN, as well as from the application of LO in pasteurized milk. To analyze the data obtained from the application of LO in raw milk, Buchanan growth curve models with no N_{\max} parameter were fit using the nlsMicrobio and nlstools packages in R. Using the confint2 function of nlstools, asymptotic 95 % confidence

intervals were calculated for the lag phase, maximum growth rate and initial concentration for every treatment. Estimates were regarded as significantly different if the 95 % confidence intervals did not overlap. In addition, an ANOVA and a Tukey's honest significant difference test were performed to compare the total microbial counts between treatments at 24 h. A count from the evaluation of the concentration of SCN^- (14 mg/L SCN^- , 0.12 LO solution, day 7) was considered an outlier based on results from previous experiments and on other three independent replicates of the same treatment.

RESULTS AND DISCUSSION

Concentration of LO

Different concentrations of a LO solution were evaluated to determine the effectiveness inhibiting the growth of *P. fragi* in skim milk. For this study, a *P. fragi* strain isolated from pasteurized milk was selected as the indicator of microbial inhibition. The LO solution was added by itself to determine if it had an antimicrobial effect due H₂O₂ production, as well as in combination with NaSCN and LP to evaluate its potential as an activator of LPS.



*Bars with different letters indicate significant difference (p<0.001) between treatments on the same day.

**For counts lower than the limit of detection, a value of 1 log₁₀ CFU/mL was used for the statistical analysis.

Figure 1. *P. fragi* counts in skim milk treated with LO and LPS during storage at (a) 6 °C and (b) 21 °C.

At 6 °C the increase in the concentration of LO caused higher reductions of *P. fragi* during 7 days of storage (Figure 1). When the LO solution was added at 0.012 g/L, *P. fragi* counts were in general lower than the control for the first 48 h of storage. However, by days 4 and 7 bacterial counts for both LO and LPS treatments were no different than the control. At this lowest LO concentration, no significant difference was determined between the addition of the oxidase alone

or combined with the LPS components by the end of the study. Samples treated with 0.12 g/L of LO showed an initial ~2.5 log reduction of *P. fragi* within 24 h followed by a slight increase of less than 1 log by day 7. The counts for this treatment were consistently lower than the control. The same bactericidal effect within 24 h was achieved when LO was used at 0.12 g/L in presence of LP and NaSCN. In addition, this treatment had a bacteriostatic effect on *P. fragi* during the remaining days of storage and counts were significantly lower compared to LO by itself on days 4 and 7. A reduction of *P. fragi* below the detection limit was achieved in 24h when the LO solution was added at 1.2 g/L no regrowth occurred after 7 days of storage. At this concentration, no significant difference was determined between the antimicrobial effect of LO added independently or combined with the LPS components. Results suggest that at this concentration of LO solution the production of H₂O₂ was enough to kill *P. fragi* below the detection limits regardless of the presence of the LPS.

None of the systems containing LO solution at 0.012 g/L had inhibitory effect on the growth of *P. fragi* at room temperature. At a concentration of 0.12 g/L, LO alone showed bacteriostatic effect for 24 h followed by regrowth to a concentration no significantly different than the control or its LPS counterpart. As observed for treatments at 6 °C, a concentration of LO solution of 1.2 g/L, exhibited bactericidal activity and *P. fragi* was reduced to undetectable concentrations within 24 h with no regrowth after 7 days.

The improved antimicrobial activity as the LO concentration increased could be caused by a higher production of H₂O₂. This compound acts as antimicrobial and as a substrate of the LPS that promotes the oxidation of SCN⁻ to OSCN⁻. Martin et al. (2014) reported that the higher the

concentration of H₂O₂ in raw milk, the greater the antimicrobial effect on total bacterial count over time during storage at 6 °C and 21 °C. Similarly, *Listeria monocytogenes* growth was only inhibited in raw milk during 21 d at a concentration of 100 ppm of H₂O₂, while at concentrations of 400 and 800 ppm counts were reduced to undetectable levels within 24 h (Kozak et al., 2017). Other studies have reported bacterial inhibition due to the production of H₂O₂ by enzymatic systems such as glucose oxidase-glucose in milk (Dionysius et al., 1992) and liquid whole egg (Dobbenie et al., 1995).

Gram-negative microorganisms such as pseudomonads can be both inhibited by the compounds produced by the LPS and killed by the H₂O₂ (De Wit and Van Hooydonk, 1996), which may explain the enhanced antimicrobial effect observed when LO was added in presence of SCN⁻ and LP. Reiter et al. (1976) studied the activation of the LPS using glucose oxidase and reported that the bactericidal effect occurred in two stages: the first one because of SCN⁻ oxidation by LP, and the second due to the accumulation of H₂O₂. This combined effect is also consistent with a previous study that showed greater antimicrobial activity against *Staphylococcus aureus*, *P. aeruginosa*, and *Bacillus cereus* for the LPS compared with H₂O₂ alone (Fweja et al., 2008). Our results also indicate that the natural concentration of lactose in milk is adequate to promote the production of H₂O₂ via LO and obtain the described antimicrobial effect.

All treatments were more effective inhibiting the growth of *P. fragi* in milk at refrigeration temperature than at room temperature (*Figure 1*), except at a concentration of 1.2 g/L of enzymatic solution. These results are in agreement with those reported by Martin et al. (2014), where H₂O₂ showed higher bacterial inhibitory effect at 6 °C than at 21 °C. Although SCN⁻ peroxidation is

higher at room temperature than at refrigeration temperatures (Adolphe et al., 2006), it is likely that the concentration of the antimicrobial product was not sufficient to inhibit growth of *P. fragi* under conditions that promote more active bacterial growth. FAO/WHO (2006) specify that the inhibitory effect of the LPS depends on the storage temperature of the milk, ranging from 5- 6 days at 4 °C and 16-17 h at 20 °C. Results from this study were consistent with those observations.

Concentration of NaSCN

The principal antimicrobial compound produced by the LPS is hypothiocyanite (OSCN^-) and it is the result of the peroxidase-catalyzed oxidation of SCN^- . This compound, known as a hypopseudo-halide, is a potent oxidant with antimicrobial activity (Bafort et al., 2014). OSCN^- oxidizes the sulphydryl groups of proteins to disulphides causing structural damage of microbial cytoplasmic membranes (Kussendrager and van Hooijdonk, 2007). The antimicrobial activity of the LPS is affected both by the concentration of SCN^- and by the method used to generate H_2O_2 since they affect the concentration of OSCN^- and the LP activity (Dionysius et al., 1992).

Based on observations from preliminary studies, we hypothesized that the level of SCN^- affects not only the activity of the LPS, but might also influence the activity of LO. Various SCN^- were evaluated to study its effect on the antimicrobial activity of LO alone and combined with LP. All treatments had bactericidal effect at 6 °C during the first 48 h and in general, there was no significant difference between samples treated with LO only or with the LPS. LO-treated samples exhibited bactericidal effect over time with counts ~ 7 log lower than the control by day 7. At NaSCN concentrations of 140 and 1400 mg/L, *P. fragi* was reduced below the detection limit after

4 d. Similarly, the activation of the LPS with 14 mg/L of NaSCN also showed bactericidal activity during 7 d of storage and counts were no significantly different compared with the LO treatments. In contrast, LPS treatments at 140 and 1400 mg/L of NaSCN exhibited regrowth on day 7. Counts for these two treatments were significantly lower than the control at every time point; however, they were significantly less effective at inhibiting growth of *P. fragi* than LO alone or with the LPS activated with 14 mg/L of NaSCN.

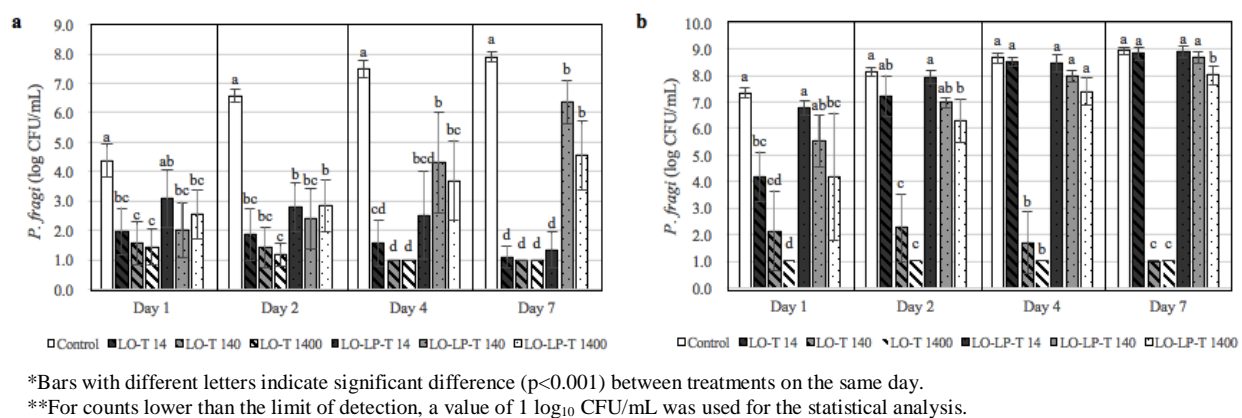


Figure 2. *P. fragi* counts in skim milk treated with LO and LPS at different concentrations of NaSCN during storage at (a) 6 °C and (b) 21 °C.

At 21 °C, the higher the concentration of SCN⁻, the greater the antimicrobial effect of the LO enzyme over storage time. The combination of SCN⁻ at 14 mg/L and LO exhibited a bacteriostatic effect for 24 h followed by regrowth of *P. fragi* to concentrations not significantly different than the control. However, LO combined with 140 and 1400 mg/L of NaSCN showed bactericidal activity. In the first case, *P. fragi* was gradually reduced over time until reaching a concentration below the detection limit on day 7. For the latter, *P. fragi* levels were reduced below the detection limits within 24 h and no regrowth was detected after 7 d. In contrast, the concentration of SCN⁻ did not have a significant effect on the antimicrobial activity of the LPS at room temperature. The

inhibition of *P. fragi* did not increase significantly with the addition of higher levels of SCN^- except on day 7 at the highest concentration, however, outgrowth was not inhibited.

Interestingly, results shown in *Figure 2* showed reduced inactivation of *P. fragi* at both temperatures when LP was present in comparison to systems with LO and SCN^- alone, particularly at increased levels of SCN^- . One explanation for the reduced antimicrobial activity of the LPS at the two higher levels of NaSCN evaluated in this study is that LP function can be inhibited by an excess level of SCN^- . The anion can bind to the native enzyme, restricting the binding site to H_2O_2 , and altering the following reactions that lead to the production of antimicrobial compounds by the LPS (Bafort et al., 2014). Dionysius et al. (1992), however, reported that increasing the concentration of SCN^- caused only a slight increase in the production of the oxidation product, and suggested that the production of H_2O_2 was the rate limiting factor in the enzymatic reaction. In both conditions, LO is producing H_2O_2 . In the LP-free conditions, H_2O_2 is the primary antimicrobial agent. If in the LP and LO containing conditions, the higher levels of SCN^- were inhibiting LP activity the non-utilized H_2O_2 would be free to act as an antimicrobial compound as in the LP-free condition and one might expect to see similar levels of inhibition, but this was not observed. These results suggest an interaction or potential synergistic effect between LO and SCN^- that improved the inactivation of *P. fragi* in absence of LP. To control for the effect of SCN^- on *P. fragi* counts, milk samples were exposed to all three different concentrations of NaSCN. Counts for NaSCN-controls were not significantly different than the control (data not shown), which means that NaSCN had no antimicrobial activity individually and reinforces the theory of a synergistic effect with LO. The influence of SCN^- on LO activity has not been studied and future research is needed to understand this potentially new mechanism of inhibition.

These results suggest that the addition of both LO and SCN^- could be studied as a potential antimicrobial system in other food matrixes. Data from this study showed that both temperature and SCN^- concentration affect the bacterial reduction achieved by the LO- SCN^- system. It should be noted that when looking to add SCN^- some studies suggest that high levels of SCN^- can interfere with iodine metabolism and reduce its uptake by the thyroid gland (FAO/WHO, 2006). However, the effect is adverse only in individuals with iodine deficiency (Laurberg et al., 2009). In addition, a concentration of NaSCN around 20 mg/L, which proved to be effective in this study, is 10-20 times lower than the level that has shown to have an effect on iodine metabolism (FAO/WHO, 2006).

Application of lactose oxidase in pasteurized milk

Pseudomonas spp. is the most common bacteria associated with post-pasteurization contamination (PPC) of fluid milk, a problem that affects approximately 50 % of the supply (Martin et al., 2018). Pasteurized skim milk was spiked with *P. fragi* and then treated with LO alone or combined with NaSCN to evaluate the interaction with the native LPS that remains active after HTST pasteurization. A maximum concentration of 0.12 g/L of LO solution was selected based on previous results (*Figure 1*) and considering the potential impact of the enzyme concentration on sensory attributes and costs. Lower concentrations were also tested to determine if there was an antimicrobial effect when applied in a commercial sample in comparison with the model systems. NaSCN was added at 14 mg/L based on results shown in *Figure 2a* and the usage recommendations by FAO/WHO (1991).

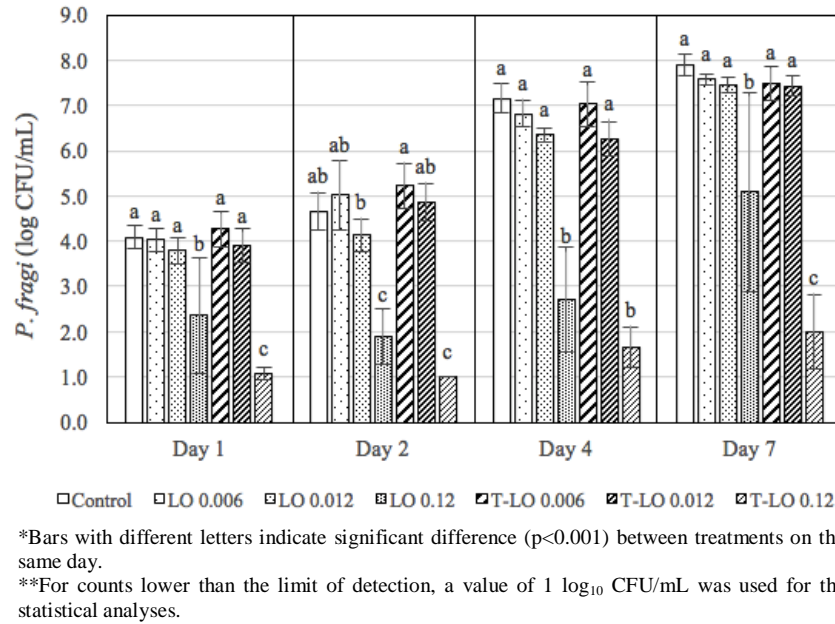


Figure 3. *P. fragi* counts in pasteurized milk treated with LO and LPS during storage at 6 °C.

Table 1. pH of pasteurized milk treated with lactose oxidase during storage at 6 °C.

TREATMENT	TIME (DAYS)		
	1	4	7
Control	6.73 ± 0.02	6.71 ± 0.03	6.71 ± 0.02
LO 0.006	6.71 ± 0.02	6.67 ± 0.01	6.65 ± 0.02
LO 0.012	6.70 ± 0.03	6.64 ± 0.02	6.60 ± 0.02
LO 0.12	6.66 ± 0.03	6.56 ± 0.02	6.44 ± 0.03
T-LO 0.006	6.71 ± 0.03	6.66 ± 0.05	6.63 ± 0.06
T-LO 0.012	6.70 ± 0.03	6.65 ± 0.03	6.60 ± 0.03
T-LO 0.12	6.65 ± 0.03	6.55 ± 0.04	6.46 ± 0.04

Figure 3 shows that concentrations of 0.006 g/L and 0.012 g/L of the LO solution caused no significant effect on *P. fragi* counts compared to the control and no significant difference was determined between adding LO with or without NaSCN supplementation. This contrasts with results shown in Figure 1, where the addition of the enzyme solution at a concentration of 0.012 g/L showed antimicrobial activity during 48 h regardless of the concentration of NaSCN. Results suggest that production of H_2O_2 at these concentrations was not sufficiently high as to activate the LPS or act as an antimicrobial. The discrepancy between results could be due to a difference

between the activity of LP present in the model systems and in the pasteurized milk. HTST pasteurization reduces up to 30 % of LP activity (De Wit and Van Hooydonk, 1996, Barret et al., 1999), which has direct impact on its antimicrobial power.

The LO solution added at 0.12 g/L reduced *P. fragi* counts within the first 24 h at both levels of NaSCN. The LO-treated milk showed an initial reduction of ~ 1.4 log and counts remained relatively constant with a difference of less than 1 log between counts for during 4 days. Regrowth occurred by day 7 but *P. fragi* counts were still significantly lower compared to the control. The addition of SCN⁻ to activate the LPS system caused a ~ 2.7-log reduction of *P. fragi* after 24 h. Counts increased ~ 1 log by day 4 and 7 but never exceeded the initial inoculum level. By day 7, the supplemented milk exhibited a ~ 6-log difference from the control and significantly lower counts compared with the rest of treatments. Supplementation with NaSCN enhanced the antimicrobial effect of the LPS and helped maintaining *P. fragi* at low levels during storage compared to the LO-treated sample. This effect was not unexpected since 8 mg/L is the average concentration of SCN⁻ in bulked milk and 15 mg/L are required for the activation of the LPS (Seifu et al., 2005, FAO/WHO, 2006).

According to the results, the remaining activity of LP after pasteurization is sufficient to achieve important microbial reductions. LO could be added to pasteurized milk to extend its shelf-life or to inhibit spoilage in case of PCC. In this study, the initial inoculum level was high and close to the Pasteurized Milk Ordinance (PMO) SPC limit of 20,000 CFU/mL. However the initial level of PCC is in general very low (Schröder, 1984) and could be inhibited by a reduced concentration of LO. FAO/WHO (2006) explain that the antimicrobial effect of the LPS is inversely correlated

to bacterial concentration. Its efficacy is low at high bacterial levels, bacteriostatic at intermediate levels, and bactericidal at low levels.

The impact of the addition of LO on the sensory attributes of milk should be noted. The production of lactobionic acid causes a decrease in the pH of milk and it could also potentially change its flavor. Table 1 shows that the lowest pH of pasteurized milk dropped to 6.46 after 7 days of storage for the most effective treatment (T-LO 0.12). However, longer storage periods or higher concentrations of enzyme might cause a more dramatic effect that impacts both sensory quality and functionality of milk. Technologies such as enzyme immobilization could be explored as an option to control the contact time between the milk and the enzyme to achieve the desired antimicrobial effect while controlling the production of acid.

Application of lactose oxidase as a raw milk preservative

The addition of LO as an activator of the LPS was compared to the reference method described in the guidelines by FAO/WHO (1991) to activate the antimicrobial system in raw milk. The FAO/WHO method consists on the addition of 14 mg/L of NaSCN and 30 mg/L of sodium percarbonate as the source of H₂O₂. Previous results from this study were used as reference and LO was added at 0.12 g/L both alone and in addition to 14 mg/L of NaSCN. Milk was stored at 21 °C and total bacterial growth monitored for 24 (*Figure 4*) to compare the results with the 16–17 h range of microbial inhibition reported by FAO/WHO (1991, 2006) at that temperature.

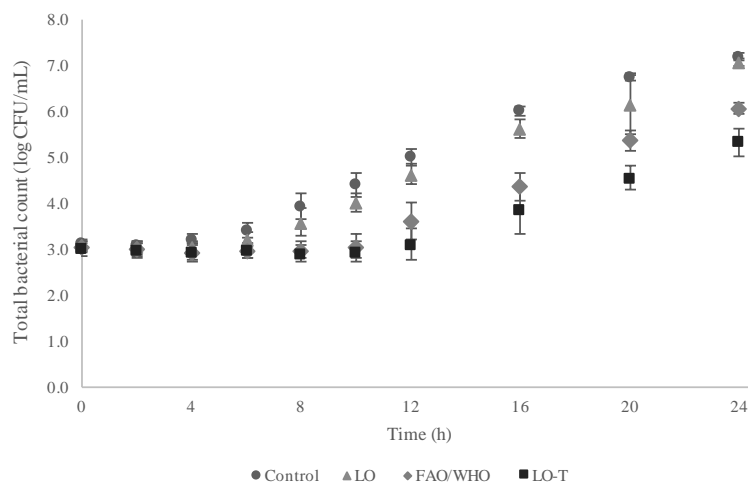


Figure 4. Total bacterial growth curve in raw milk stored at 21 °C.

Table 2. Parameters determined from the total bacterial growth curve in raw milk stored at 21 °C.

TREATMENT	LAG PHASE* (H)	MAX GROWTH RATE (H ⁻¹)	24-H COUNT** (LOG CFU/ML)	24-H PH
CONTROL	4.0 ± 1.0 ^a	0.50 ± 0.04	7.21 ± 0.07 ^c	6.68 ± 0.01
FAO/WHO	9.4 ± 1.0 ^b	0.49 ± 0.05	6.09 ± 0.12 ^b	6.72 ± 0.01
LO	5.4 ± 0.9 ^a	0.51 ± 0.03	7.06 ± 0.08 ^c	6.66 ± 0.01
LO-T	11.3 ± 1.4 ^b	0.42 ± 0.07	5.34 ± 0.30 ^a	6.67 ± 0.01

* Means with different letters indicate significant difference (p<0.05) between treatments.

**Means with different letters indicate significant difference (p<0.0001) between treatments.

The average lag phase was significantly longer for the samples treated with both LO and SCN⁻ than the control and LO alone, but not significantly different from the reference method (*Table 2*).

The average maximum growth rate during the exponential phase was 0.48 h⁻¹, and no significant difference was determined among treatments. However, the total bacterial count after 24 h for the LO+SCN-treated sample was significantly different lower compared to the other treatments. The final count was ~ 1.8-log lower than the control and LO added alone, and ~ 0.8 log lower compared to the reference method. This is consistent with reports that mention that the production of H₂O₂ by enzymatic systems such as glucose oxidase/glucose, and xanthine oxidase/hypoxanthine may

improve the antimicrobial activity of the LPS compared to added H_2O_2 (Kussendrager and van Hooijdonk, 2007). According to Dionysius et al. (1992), the $\text{H}_2\text{O}_2/\text{OSCN}^-$ ratio is low when an enzymatic method is used to generate H_2O_2 and that promotes OSCN^- as the main oxidation product. When the ratio is high, excess H_2O_2 oxidizes OSCN^- leading to the formation of other compounds with no antimicrobial activity. This could explain the enhanced antimicrobial activity achieved when the LO-lactose system was used as the activation method. It should be noted that LO activated the LPS and microbial growth was inhibited regardless the higher fat content of this sample in contrast with skim milk samples used in previous experiments.

Consistently with previously discussed results, supplementation with NaSCN improved the antimicrobial effect of the LPS. Probably, the concentration of SCN^- in the milk was not sufficient to activate the LPS and therefore the addition of LO alone showed no significant difference in the evaluated parameters compared to the control. Overall, the LPS activation method adding LO and SCN^- showed the most potential for preservation of raw milk stored under abused temperature and its inhibition effect is comparable to the method of reference.

An advantage of the application of LO as an activator of the LPS is that lactose, its substrate, is naturally present in milk. On the contrary, the use of other enzymatic systems might imply the addition of excess sugars such as glucose. Another promising result is that, as shown in *Table 2*, after 24 h the pH for the sample treated with LO and NaSCN was still around 6.7, which is the normal value for fresh milk (Walstra et al., 2005). Since the activation of the LPS does not substitute pasteurization (FAO/WHO, 1991), heat treating the raw milk would cause the inactivation of LO and thus stop further production of acid.

CONCLUSION

Results from this study suggest that LO can act as an alternative source of H_2O_2 that enhances the microbial inhibition achieved by the LPS, and may work synergistically with SCN^- in the absence of LP. LO could be used to create enzyme-based preservation technologies for applications in locations where cold chain access is limited. Additionally, LO proved to be effective under refrigeration conditions, showing that it could be used as a preservative in situations where raw milk needs to be held for extended periods of time before pasteurization and further processing. Additional studies should address methods, such as enzyme immobilization, to prevent pH drop in the milk for applications of LO in final products like pasteurized milk. Finally, since enzymes are present in diverse dairy foods, the application of LO as an antimicrobial represents an opportunity for the dairy industry to benefit from an extended the shelf-life of their products while meeting the consumer desire for clean labels.

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CHAPTER 2

INHIBITION OF FOODBORNE PATHOGENS AND SPOILAGE MICROORGANISMS ASSOCIATED WITH DAIRY PRODUCTS BY LACTOSE OXIDASE

ABSTRACT

The inhibitory effect of lactose oxidase on the growth of foodborne pathogens and spoilage microorganisms associated with dairy products was evaluated through an overlay inhibition assay. Lactose oxidase generates hydrogen peroxide via lactose oxidation into lactobionic acid. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* Ser. Typhimurium, *Staphylococcus aureus*, *Pseudomonas fragi*, and *Penicillium chrysogenum* were used as indicators. A commercially available solution of lactose oxidase was applied at different concentrations (0, 0.12, 1.2, and 12 g/L) in four types of media (BHI, BHI+NaSCN, BHI+lactose, and BHI+NaSCN+lactose) to evaluate the effect of lactose and SCN⁻ on the microbial inhibition. LO inhibited the growth of all the indicators at a concentration of 12 g/L of the enzyme solution in the presence of lactose alone and in combination with NaSCN. However, supplementation with NaSCN had no effect on the magnitude of the microbial inhibition. *S. aureus* was the most sensitive pathogen and *P. fragi* was the most sensitive of all the indicators in general. *L. monocytogenes* and *P. fragi* showed higher susceptibility to the antimicrobial effect of LO at 6 °C than at their corresponding optimum growth temperature. The inhibitory effect was attributed to the generation of hydrogen peroxide from the oxidation of lactose by LO. Findings from this study demonstrate that LO could be used as a novel approach to inhibit the growth of mold and bacteria. It could also be applied as a label-friendly preservative not only in dairy foods but also in products supplemented with lactose.

INTRODUCTION

Foodborne pathogens cause around 9.4 million illnesses; 56,000 hospitalizations; and 1,350 deaths in the United States each year (CDC, 2016). Specifically, dairy products were attributed 657,000 of the foodborne illnesses caused by pathogenic bacteria between 1998 and 2008 (Painter et al., 2013). Some of the prevalent pathogens associated with dairy foods are *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter* spp., and *Staphylococcus aureus* (Lu and Wang, 2017). The presence of these pathogens in dairy products can be due to a faulty pasteurization, contaminated processing environment and equipment, and poor hygiene practices of food handlers (Oliver et al., 2005, Fernandes, 2009). For example, grated Parmesan cheese was recalled because of potential contamination with *Salmonella* (FDA, 2016), *S. aureus* and *L. monocytogenes* have been the cause of Queso Fresco recalls, and contamination with *L. monocytogenes* caused an ice cream recall (CDC, 2018).

Food loss is another major issue for the dairy industry and microbial spoilage is one of the main contributors (Sperber, 2009). More than 24 billion pounds of dairy products went to waste in 2010 and dairy was the food group with the largest loss at the retail level (Buzby et al., 2014). Common sources of spoilage microflora are raw milk; water, air and surfaces in contact with the product; and brines (Ledenbach and Marshall, 2009). Psychrotrophic bacteria represent the main cause of spoilage of dairy products and *Pseudomonas* spp. is one of the predominant species due to their ability to grow under refrigeration conditions (Lu and Wang, 2017). *Pseudomonas* growth causes off-flavors and off-odors due to the production of enzymes that degrade proteins and lipids

(Ledenbach and Marshall, 2009), as well as color changes in the surface of dairy products like cheese (Martin et al., 2011).

Molds are also a common cause of spoilage dairy products such as cheese and yogurt. Molds can grow under acidic conditions in fermented dairy products where bacterial growth is inhibited and cause visual surface changes in color, odor, and flavor (Lu and Wang, 2017). *Penicillium* spp. is the most commonly reported spoilage genus (Garnier et al., 2017) and it has been found in vacuum-sealed cheese stored under refrigeration (Lu and Wang, 2017). Studies have shown that some *Penicillium* strains are resistant and able to metabolize sorbate, a common cheese preservative, and develop off-odors and off-flavors (Ledenbach and Marshall, 2009, Garnier et al., 2017).

The implementation of good manufacturing practices and the application of technologies such as pasteurization, air filtration, and modified atmospheres has reduced both spoilage and the number of outbreaks caused by dairy products. Nevertheless, post-processing contamination remains an issue for the food industry and there is a need for technologies to control it (Boor et al., 2017, Garnier et al., 2017). Preservatives such as sorbic acid, benzoic acid, and acetic acid, as well as antibiotics such as nisin and natamycin have aided the dairy industry in preventing bacteria and mold growth during storage (Herr, 2011). However, with the increasing consumer demand for clean labels and “natural” products, it is a challenge for the industry to prevent spoilage and pathogen growth without adding preservatives.

Enzyme-based preservation technologies represent a clean label approach to preserve dairy foods. Enzymes are already part of their ingredient list since they are widely used in dairy applications such

as milk coagulation and cheese ripening. Lactose oxidase (LO) generates hydrogen peroxide, a well-known antimicrobial compound, as a product of the oxidation of lactose to lactobionic acid (Nordkvist et al., 2007). Hydrogen peroxide has proven to be an effective antimicrobial in foods such as leafy greens (Moore et al., 2011), milk (Martin et al., 2014, Kozak et al., 2017), and queso fresco (Kozak et al., 2018), among others. However, the addition of hydrogen peroxide is not aligned with current consumer trends and is not always feasible or permitted depending on the product.

In a previous study, LO showed bactericidal effect against *Pseudomonas fragi* in milk and a synergistic effect with thiocyanate (SCN^-). However, its antimicrobial activity against foodborne pathogens and other spoilage microorganisms like molds has not been investigated. The objective of this study was to evaluate the antimicrobial effect of LO against pathogens and spoilage microorganisms associated with milk and dairy products.

MATERIALS AND METHODS

Indicator strains

Four foodborne pathogens, one spoilage microorganism and one mold (*Table 3*) were used to determine the antimicrobial activity of LO (LactoYIELD®, Chr. Hansen, Milwaukee, WI) in different media. For all the bacterial strains, a frozen culture was streaked onto brain heart infusion agar (BHI; Difco, BD Diagnostics, Franklin Lakes, NJ) and incubated for 24 h at 37 °C for pathogens and 32 °C for *P. fragi*. An individual colony was then used to inoculate 5 mL of BHI broth, followed by 18 h of incubation at the same temperatures previously mentioned. The mold was spot plated onto Malt Extract Agar (Difco, BD Diagnostics, Franklin Lakes, NJ) and incubated at 25 °C for 4 weeks. Spore formation was confirmed by microscopy. Spores were harvested by adding sterile water with 0.1% Tween 80 (Baker Analyzed, Philipsburg, NJ) to the plate and scraping the wet surface with a sterile spreader. The spores and mycelia were filtered through sterile cheesecloth into a sterile bottle to isolate spores only. The suspensions were combined with glycerol at 50 % (v/v) and stored at -80 ± 2 °C until use.

Table 3. Classification and growth temperatures for microorganisms used as indicators

Microorganism	Classification	Incubation temperature (°C)
<i>Escherichia coli</i> O157:H7 (ATCC 43889) ^a	Pathogenic	37
<i>Listeria monocytogenes</i> ^b	Pathogenic	37
<i>Salmonella enterica</i> Ser. Typhimurium (ATCC14028) ^b	Pathogenic	6 and 37
<i>Staphylococcus aureus</i> (ATCC 8095) ^a	Pathogenic	37
<i>Pseudomonas fragi</i> ^b	Spoilage	6 and 32
<i>Penicillium chrysogenum</i> ^a	Spoilage	25

^a From the Food Safety Laboratory, Cornell University, Ithaca, NY

^b From the collection of Dr. Randy Worobo's, Cornell University, Geneva, NY

Overlay inhibition assay

Four different types of media were used to test the inhibitory effect of lactose oxidase: BHI, BHI supplemented with NaSCN (14 mg/L), BHI supplemented with lactose (2.5 % m/v), and BHI supplemented with both NaSCN (14 mg/L) and lactose (2.5 % m/v). NaSCN and lactose were added to the media after sterilization in a solution previously filter-sterilized through a SFCA 0.20 μm pore size. The well diffusion method described by Ahn and Stiles (1990) was followed to test the antimicrobial effect of four different concentrations (0, 0.12, 1.2, and 12 g/L) of the lactose oxidase solution. Wells (8 mm-diameter) cut into each type of media were filled with 100 μL of enzyme solution and allowed to diffuse into the agar in a laminar flow hood. Once the solution had been completely absorbed, a 5 μL aliquot of catalase (Sigma Aldrich, St. Louis, MO) was spotted next to the well to determine if the antimicrobial effect was caused by hydrogen peroxide. Plates were overlaid with a 10-mL aliquot of 0.75 % agar inoculated with 100 μL of 18-h culture for bacteria or spore suspension. Plates were incubated at 37 °C for all the pathogens, at 32 °C for *P. fragi*, and at 6 °C for *L. monocytogenes* and *P. fragi* because of their ability to grow under refrigerated conditions. For *P. fragi*, the assay was replicated using Luria-Bertani (LB) agar (Difco, BD Diagnostics, Franklin Lakes, NJ). The antimicrobial activity was determined by measuring the width of the inhibition zone. Three perpendicular measurements of the diameter per well were used to calculate the average inhibition diameter for each treatment. Assays were carried out in duplicate and repeated three times.

RESULTS AND DISCUSSION

The antimicrobial activity of a LO solution was evaluated by applying increasing concentrations of the enzyme on media with different composition to evaluate the effect of lactose and SCN^- on microbial growth inhibition. Results shown in *Table 4* show that growth of all the indicators was inhibited when the LO solution was applied at a concentration of 12 g/L in presence of lactose alone and combined with NaSCN. Among the pathogens, *S. aureus* was found to be the most sensitive as it was the only species in the study that was inhibited at a concentration of 1.2 g/L of LO solution under optimal growth conditions.

To determine if the generation of H_2O_2 was the cause of the antimicrobial activity, catalase was spotted next to the well to remove the compound where the inhibition zone would be formed. Results showed microbial growth on the area where the catalase was located for all the indicators (see Appendix). Based on these observations, it was concluded that the antimicrobial activity was attributed to the production of H_2O_2 as a product of lactose oxidation by LO. Other studies have attributed an antimicrobial effect to the production of hydrogen peroxide by lactobacilli (Price and Lee, 1970, Collins and Aramaki, 1980) and glucose oxidase present in honey (Mundo et al., 2004).

With the exception of *P. fragi*, none of the microorganism in this study were inhibited on the control or the NaSCN-supplemented media at any level of LO, showing that the antimicrobial effect is mainly caused by the production of H_2O_2 *in situ* due to the oxidation of lactose, and not by any of the components of the enzymatic solution. Also, based on the standard deviations shown in *Table 4*, it can be argued that the addition of NaSCN had no influence on the antimicrobial effect

of the LO activity. This result contrasts previous results obtained in milk, where SCN^- enhanced the antimicrobial activity of LO against *P. fragi*. This difference may be attributable to differences in the distribution of the LO, homogenous in the milk vs diffusion in the agar, and the differences in the application and level of the bacterial load between the two studies.

Table 4. Inhibition of growth of foodborne pathogens and spoilage microorganisms by lactose oxidase on BHI with different supplementations

Indicator	LO solution (g/L)	Incubation temperature (°C)	Zone of inhibition (mm)			
			Control	NaSCN	Lactose	NaSCN + Lactose
<i>E. coli</i>	0.12	37	NI	NI	NI	NI
	1.2	37	NI	NI	NI	NI
	12	37	NI	NI	3.9 ± 0.6	3.6 ± 0.4
<i>L. monocytogenes</i>	0.12	6	NI	NI	NI	NI
	1.2	6	NI	NI	3.4 ± 0.3	4.1 ± 0.7
	12	6	NI	NI	10.6 ± 0.6	9.4 ± 2.4
	0.12	37	NI	NI	NI	NI
	1.2	37	NI	NI	NI	NI
	12	37	NI	NI	3.4 ± 0.4	3.1 ± 0.6
<i>S. aureus</i>	0.12	37	NI	NI	NI	NI
	1.2	37	NI	NI	1.7 ± 0.9	1.8 ± 0.4
	12	37	NI	NI	5.4 ± 0.6	5.1 ± 0.7
<i>S. enterica</i> Ser. Typhimurium	0.12	37	NI	NI	NI	NI
	1.2	37	NI	NI	NI	NI
	12	37	NI	NI	4.5 ± 1.1	4.6 ± 0.4
<i>P. fragi</i>	0.12	6	NI	NI	2.9 ± 0.4	3.5 ± 0.5
	1.2	6	4.9 ± 0.1	4.1 ± 0.4	5.8 ± 0.5	6.5 ± 0.3
	12	6	7.7 ± 0.3	6.5 ± 0.4	8.0 ± 0.5	9.0 ± 0.8
	0.12	32	NI	NI	NI	NI
	1.2	32	2.9 ± 0.8	2.8 ± 0.8	5.5 ± 0.3	5.4 ± 0.2
	12	32	7.1 ± 0.2	6.5 ± 0.3	7.4 ± 1.8	9.3 ± 0.8
<i>P. chrysogenum</i>	0.12	25	NI	NI	NI	NI
	1.2	25	NI	NI	NI	NI
	12	25	NI	NI	4.6 ± 0.1	5.2 ± 0.6

NI: no inhibition

At 6 °C, *P. fragi* and *L. monocytogenes* showed higher sensitivity to the antimicrobial effect of LO than at their optimum growth temperature. *L. monocytogenes* was inhibited at a lower enzyme concentration (1.2 g/L) and the zone of inhibition at 12 g/L tripled. *P. fragi* was inhibited at the lowest concentration of LO solution (0.12 g/L). These findings are promising for applications of LO as a preservative in refrigerated products, since *P. fragi* and *L. monocytogenes* have the ability to grow at low temperatures and are one of the main causes of food spoilage (Ledenbach and Marshall, 2009) and recalls (CDC, 2018), respectively.

As mentioned before, *P. fragi* was the only indicator inhibited on the control (BHI agar) and on NaSCN-supplemented media. Since bacterial growth was observed in the area where the catalase was spotted (Appendix, *Figure 9* and *Figure 10*), the inhibition could potentially be attributed to the H₂O₂ produced from the oxidation of glucose, one of the components of BHI agar. Even though LO has high specificity for lactose as substrate, it is also able to oxidase other saccharides such as glucose, galactose, maltose, and cellobiose (Nordkvist et al., 2007). To confirm this effect, the overlay inhibition assay was replicated using LB agar since it does not contain any of those saccharides, and thus would preclude H₂O₂ production. On this media, *P. fragi* was not inhibited at any level of LO (Appendix, *Figure 11*) and thus supporting the conclusion that H₂O₂ production from the oxidation of glucose by LO was source of the inhibition observed in absence of lactose. The inhibition of *Pseudomonas* due to oxidation of a saccharide different than lactose propose that LO could be used as a preservative in food matrices with different sugar profiles where this particular spoilage organism is of concern.

LO also showed inhibition of *P. chrysogenum* growth in both media containing lactose. Other studies have reported antifungal activity of hydrogen peroxide against food spoilage molds like *Penicillium expansum* (Venturini et al., 2002) and *Penicillium digitatum* (Cerioni et al., 2009). It is important to mention that *P. chrysogenum* was also inhibited at 6 °C on both media containing lactose, however, it was not possible to measure a zone of inhibition because inhibition zones from different treatments merged (data not shown). As with *P. fragi* and *L. monocytogenes*, these results are promising for the application of LO as a preservative in refrigerated foods such as dairy products.

CONCLUSION

The broad antimicrobial activity of LO against foodborne pathogens and spoilage mold and bacteria, even under refrigerated conditions, suggest that LO could be applied as a novel food preservative in dairy products. Further investigation is required to develop applications considering the composition and processing of the product of interest. It is also necessary to assess the impact of LO on the sensory properties of the product. In addition, the microbial inhibition of LO against undesirable microorganisms associated other foods should be studied, as well as the impact of saccharides different than lactose on its antimicrobial activity.

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CHAPTER 3

OUTLOOKS AND PERSPECTIVES

- In the first stages of the project, we tried to characterize lactose oxidase activity at different concentrations of both enzyme and lactose. Measurements were performed using an enzymatic assay kit (Fluorimetric Hydrogen Peroxide Assay Kit, Sigma Aldrich). Results from those experiments were not considered accurate enough to report them because of hydrogen peroxide being so unstable and because the lactose oxidation reaction was being carried out in presence of different reagents from the assay kit. However, results were used as reference to define the concentrations of lactose oxidase to be used in further experiments.

- The application of lactose oxidase as an inhibitor of *Listeria monocytogenes* in queso fresco was tested dipping previously inoculated cheese in solutions with different concentrations of lactose oxidase for 1 minute. Cheese was then vacuum sealed in plastic bags (Food Saver) and stored under refrigeration. The enzymatic treatment was not effective probably due to lack of oxygen necessary for lactose oxidase activity. However, other factors that could potentially affect the results are concentration of lactose in the cheese, concentration of lactose oxidase, and dipping time. Other application methods such as coatings or incorporating the enzyme at some point during cheese processing could be studied.

- The effect of lactose oxidase as a brine preservative should be further studied. Preliminary experiments showed antibacterial activity against *Listeria monocytogenes* in commercial Feta cheese brines even without lactose supplementation. However, it showed no activity

in a commercial Mozzarella cheese brine. We suggest performing studies with brines prepared in the laboratory to control for their composition and other variables that may affect the effectiveness of the enzyme as an antimicrobial.

- Based on results from this study, the concentration of NaSCN should be considered in the development of further applications of lactose oxidase. Also, this compound could be added as a vegetable extract or as a sub-product of milk ultrafiltration to prevent a negative impact on the list of ingredients of the final product; given the current trend for clean-label products.
- Lactose oxidase immobilization can be studied as a solution to the pH drop caused by the enzyme during shelf-life in pasteurized milk. A possibility is to immobilize the enzyme on a surface and pass the milk through it before pasteurization. In this way, the antimicrobial effect by hydrogen peroxide and by the activation of the LPS could be achieved preventing migration of the enzyme to the final product. In addition, in case of enzyme migration to the milk, it would be inactivated with subsequent pasteurization.
- The application of lactose oxidase as a preservative could be studied in non-dairy products since it showed antimicrobial activity as a result of glucose oxidation. Potential applications could be for example, acidified beverages or fruit juices.

APENDIX

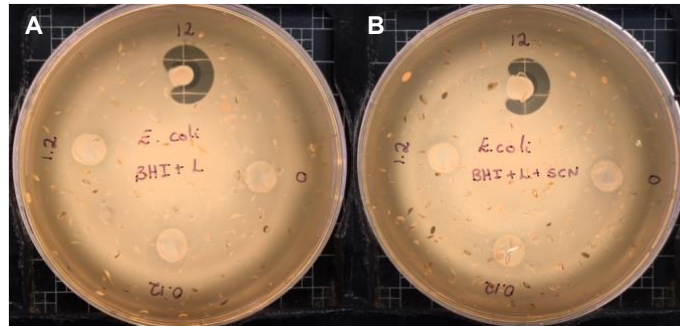


Figure 5. Inhibition of *Escherichia coli* by lactose oxidase at 37 °C on BHI agar with (A) 2.5 % lactose and (B) 2.5 % lactose and 14 mg/L NaSCN.

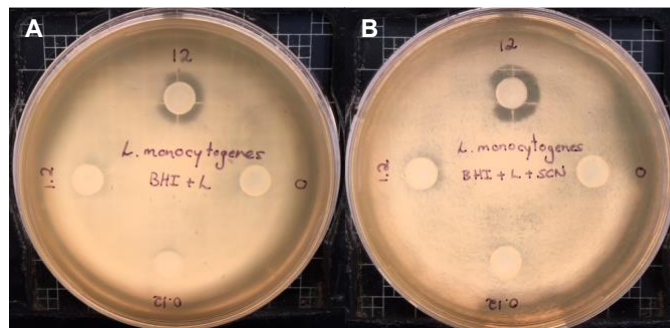


Figure 6. Inhibition of *Listeria monocytogenes* by lactose oxidase at 37 °C on BHI agar with (A) 2.5 % lactose and (B) 2.5 % lactose and 14 mg/L NaSCN.

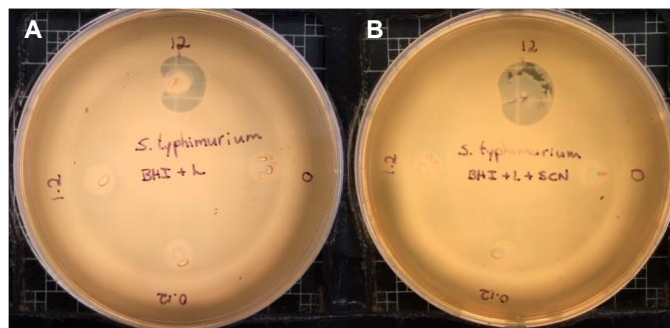


Figure 7. Inhibition of *Salmonella enterica* Ser. Typhimurium by lactose oxidase at 37 °C on BHI agar with (A) 2.5 % lactose and (B) 2.5 % lactose and 14 mg/L NaSCN.

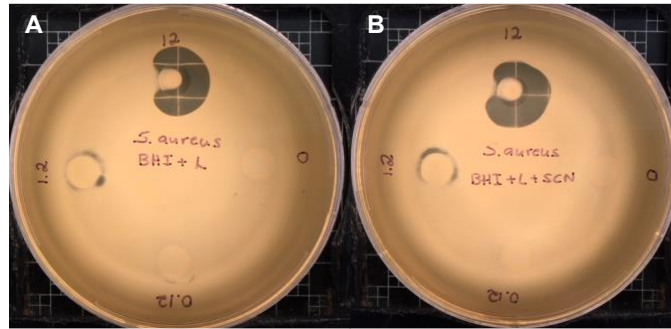


Figure 8. Inhibition of *Staphylococcus aureus* by lactose oxidase at 37 °C on BHI agar with (A) 2.5 % lactose and (B) 2.5 % lactose and 14 mg/L NaSCN.

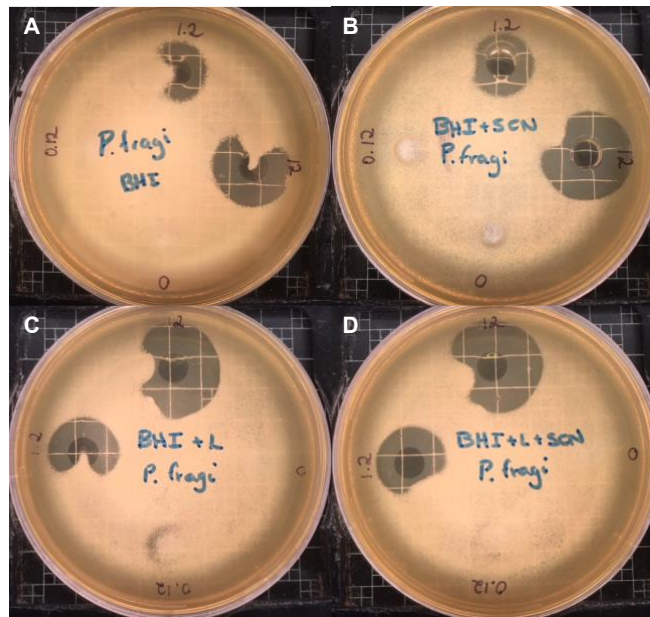


Figure 9. Inhibition of *Pseudomonas fragi* by lactose oxidase at 32 °C on BHI agar with (A) no supplementation (control) (B) 14 mg/L NaSCN (C) 2.5 % lactose and (D) 2.5 % lactose and 14 mg/L NaSCN.

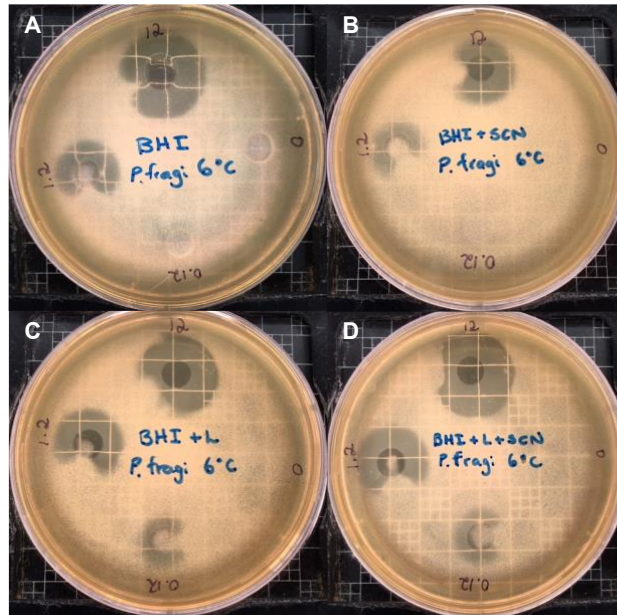


Figure 10. Inhibition of *Pseudomonas fragi* by lactose oxidase at 6 °C on BHI agar with (A) no supplementation (control) (B) 14 mg/L NaSCN (C) 2.5 % lactose and (D) 2.5 % lactose and 14 mg/L NaSCN.



Figure 11. *Pseudomonas fragi* on LB agar treated with lactose oxidase and incubated at 32 °C.

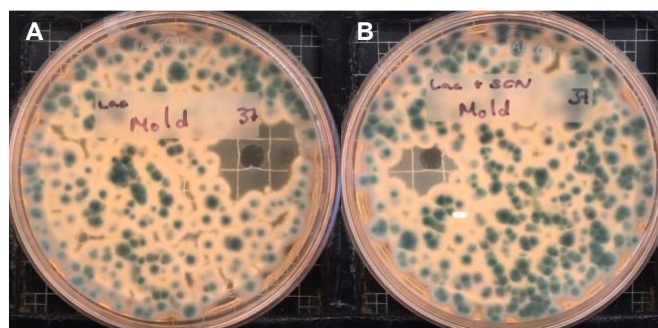


Figure 12. Inhibition of *Penicillium chrysogenum* by lactose oxidase at 37 °C on BHI agar with (A) 2.5 % lactose and (B) 2.5 % lactose and 14 mg/L NaSCN.